

## Epizootic Hemorrhagic Disease: Analysis of Tissues by Amplification and In Situ Hybridization Reveals Widespread Orbivirus Infection at Low Copy Numbers

SCOTT J. BRODIE,<sup>1,2\*</sup> KATHERINE D. BARDSLEY,<sup>1</sup> KURT DIEM,<sup>2</sup> JAMES O. MECHAM,<sup>1</sup>  
SCOTT E. NORELIUS,<sup>3</sup> AND WILLIAM C. WILSON<sup>1</sup>

*Arthropod-Borne Animal Disease Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Laramie, Wyoming 82071<sup>1</sup>; Virology Division/Retrovirology Laboratory, University of Washington School of Medicine, Seattle, Washington 98144<sup>2</sup>; and Wyoming Game and Fish Department, Sundance, Wyoming 82729<sup>3</sup>*

Received 27 June 1997/Accepted 29 January 1998

**A recent outbreak of hemorrhagic fever in wild ruminants in the northwest United States was characterized by rapid onset of fever, followed shortly thereafter by hemorrhage and death. As a result, a confirmed 1,000 white-tailed deer and pronghorn antelope died over the course of 3 months. Lesions were multisystemic and included severe edema, congestion, acute vascular necrosis, and hemorrhage. Animals that died with clinical signs and/or lesions consistent with hemorrhagic fever had antibody to epizootic hemorrhagic disease virus serotype 2 (EHDV-2) by radioimmune precipitation but the antibody was limited exclusively to class immunoglobulin M. These findings, indicative of acute infection, were corroborated by the observation that numerous deer were found dead; however, clinically affected deer were rarely seen during the outbreak. Furthermore, only in animals with hemorrhagic lesions was EHDV-2 isolated and/or erythrocyte-associated EHDV-2 RNA detected by serotype-specific reverse transcription (RT)-PCR. By using a novel RT in situ PCR assay, viral nucleic acid was localized to the cytoplasm of large numbers of tissue leukocytes and vascular endothelium in tissues with hemorrhage and to vessels, demonstrating acute intimal and medial necrosis. Because PCR amplification prior to in situ hybridization was essential for detecting EHDV, the virus copy number within individual cells was low, <20 virus copies. These findings suggest that massive covert infection characterized by rapid dissemination of virus facilitates the severe and lethal nature of this disease.**

Epizootic hemorrhagic disease viruses (EHDV) are one of 13 serogroups in the genus *Orbivirus*, family *Reoviridae*. All members of this genus replicate in the cytoplasm of infected cells (12) and have a double-layered protein capsid consisting of seven polypeptides, each of which is encoded by one of 10 double-stranded RNA viral segments (12, 34). Two serotypes of EHDV are found in North America. EHDV serotype 1 (EHDV-1) and EHDV serotype 2 (EHDV-2) are enzootic in white-tailed deer in the southeastern United States, where they generally have little or no effect on their principle host (36). In contrast, disease outbreaks associated with high morbidity and mortality, and affecting large populations of white-tailed deer, occur periodically in the Western states in association with predominantly EHDV-2. This apparent disparity in viral pathogenesis has been attributed, at least in part, to differences in virulence of viral serotypes, increased host susceptibility in nonenzootic areas, and geographic variance in vector competency (45, 48, 49, 55).

Various strategies based on PCR have been used to detect orbiviral RNA (1, 2, 57). Because orbiviruses nonspecifically bind erythrocyte cell surface glycoproteins with high affinity, erythrocyte lysates have been used for diagnostic procedures utilizing PCR (5, 32, 47). In situ hybridization has also been used with relative success to detect orbivirus-infected cells in culture (11). However, attempts to localize orbiviral nucleic acids and protein antigens in vivo have generally been unrewarding (16, 17, 53). These findings have been attributed to

low levels of virus replication and antigen expression in vivo. In other viral systems, quantitative PCR has been used to detect rare target sequences (43); however, with this technique the association with individual cells is lost. Although conventional in situ hybridization will identify target sequence in a single cell, a low-copy-number target sequence may not be detected. The combination of PCR with in situ hybridization allows the target sequence to be amplified above the limit of detection (3, 21, 23, 38).

The primary objective of this study was to apply a novel reverse transcription (RT) in situ PCR strategy to clinical samples obtained from wild ruminants naturally infected with EHDV and second, to describe pathogenetic mechanisms of EHDV as it related to the 1995 epizootic, which killed an estimated 25,000 to 50,000 white-tailed deer (>1,000 laboratory-confirmed cases) in northeast Wyoming and adjacent South Dakota, North Dakota, and Montana (36a). Examination of tissues by in situ hybridization and RT in situ PCR revealed covert, yet massive, infection of mononuclear leukocytes and of endothelial cells in sites of acute vascular necrosis and hemorrhage.

### MATERIALS AND METHODS

**Animals and sample preparation.** Biological samples were collected from white-tailed deer, from mule deer, and from pronghorn antelope that had recently died or were captured with signs of hemorrhagic fever (Table 1). In addition, clinically normal deer ( $n = 3$ ), domestic sheep ( $n = 24$ ), and cattle ( $n = 12$ ), from regions within the EHDV epizootic, were examined serologically for orbivirus infection. Peripheral blood and/or heart blood, pericardial fluid, and selected tissues were collected from all animals within 1 to 4 h of death and included bone marrow, coronary band and orofacial skin, skeletal muscle from the neck and tongue, right frontal cerebral cortex, cerebellum, brain stem, spinal cord at the level of the second cervical vertebra, right caudal lung lobe (including pulmonary artery), trachea, tonsil, sternal and mediastinal lymph nodes, heart,

\* Corresponding author. Present address: University of Washington School of Medicine, Department of Laboratory Medicine, Vaccine/Virology Division, Room T293X, Seattle, WA 98195. Phone: (206) 685-6894. Fax: (206) 685-3639. E-mail: sjbrodie@u.washington.edu.

TABLE 1. Clinicopathologic findings

Animal no. <sup>a</sup>	Species <sup>b</sup>	Sex <sup>c</sup>	Age (yr) <sup>d</sup>	Clinical signs <sup>e</sup>	Principal lesions <sup>f</sup>
1	WTD	M	1	Found dead	Blood in thoracic cavity and pericardial sac
2	WTD	M	5	Severe four-limb lameness; vesicles on oronasal mucosa and coronary band; lingual erosions; euthanized	Multifocal pulmonary and cerebral hemorrhage
3	WTD	M	1	Found dead	Blood in thoracic cavity, pericardial sac, and abdomen
4	WTD	M	1	Severely disoriented, depressed, and bleeding from the oronasal cavity; euthanized	Multifocal pulmonary and cerebral hemorrhage; acute vascular necrosis and thrombosis
5	A	F	4	Severely depressed and bleeding profusely from the oronasal, rectal, and vaginal cavities; euthanized	Severe multicentric hemorrhage; acute disseminated vascular necrosis and thrombosis
6	A	F	3	Found dead	Blood in thoracic and pericardial cavities
7	WTD	F	4	Unable to stand and bleeding from the oral, nasal, and rectal cavities; died during capture	Severe multicentric hemorrhage; acute vascular necrosis and thrombosis
8	WTD	M	2	Clinically normal; euthanized	None
9	WTD	F	4	Clinically normal; euthanized	None
10	WTD	F	7	Clinically normal; euthanized	None

<sup>a</sup> Animals 1 to 7 had confirmed EHDV-2 infection by laboratory analyses (Table 2). Animals 8 to 10 represented clinically healthy, lesion-free, EHDV-negative animals from the affected area.

<sup>b</sup> WTD, white-tailed deer; A, antelope.

<sup>c</sup> M, male; F, female.

<sup>d</sup> Estimated by dental arcade.

<sup>e</sup> Time of death in animals that were found dead was estimated by body temperature and carcass condition. Only animals dead for  $\leq 4$  h were further studied. Euthanasia was performed on moribund animals (no. 2, 4, 5, and 7) by intravenous overdose of pentobarbital (100 mg/kg) or by gun shot to the chest (no. 8 to 10).

<sup>f</sup> Lesions associated with disseminated microvascular changes.

spleen, kidney, liver, urinary bladder, suprascapular and mesenteric lymph nodes, rumen, abomasum, and small and large bowel. Body fluids were collected aseptically into 5-ml Vacutainer tubes containing K<sub>2</sub>EDTA. For peripheral blood, plasma was removed from cells by centrifugation and saved frozen at  $-80^{\circ}\text{C}$  until use. Blood cells were then added to an equal volume of buffered lactose peptone medium and stored at  $4^{\circ}\text{C}$  for preservation of virus infectivity. Tissues were fixed for a maximum of 48 h in 10% neutral buffered formalin for routine histopathology and in Streck tissue fixative (Streck Laboratories, Inc., Omaha, Neb.) for localization of viral nucleic acid. Paraffin-embedded tissues were sectioned (5  $\mu\text{m}$ ), mounted on silane (3-aminopropyltriethoxysilane; Sigma, St. Louis, Mo.)-treated glass microscope slides (two serial sections per slide), and examined for microscopic lesions and cell-associated viral nucleic acid by in situ hybridization and RT in situ PCR. In addition, selected tissues were snap-frozen in O.C.T. compound (Miles Inc., Elkhart, Ind.) for detection of viral antigens by immunohistochemistry.

A variety of diagnostic laboratory procedures, including virus isolation, bacteriology, and histopathology, were used to investigate the potential role of other agents during this outbreak. All animals were seronegative and/or negative by the appropriate virus isolation technique for bovine virus diarrhea virus, infectious bovine rhinotracheitis virus, and bovine and ovine adenovirus. In addition, lung samples were negative by culture for *Pasteurella* spp., and routine pathology showed no evidence of malignant catarrhal fever, including generalized lymphocytic vasculitis, lymphocytic meningitis, or corneal edema.

**Erythrocyte sample preparation for PCR.** Peripheral blood mononuclear cells (PBMC) and platelets were separated from erythrocytes by density gradient centrifugation on Histopaque (Sigma) as described previously (6, 7). PBMC were examined for viral antigens by immunocytochemistry, erythrocytes ( $10^7$ ) were lysed in sterile water (10 ml of H<sub>2</sub>O,  $37^{\circ}\text{C}$ , 20 min), and viral RNA was extracted from membranes by using phenol-chloroform-isoamyl alcohol (25:24:1; United States Biochemical, Cleveland, Ohio). The cell lysates were used for a variety of PCR-based procedures.

**EHDV-specific PCR.** Serotype-specific RT-PCR for EHDV gene segment 2 was used to distinguish EHDV-1 from EHDV-2 (1, 2). Briefly, erythrocyte-associated viral RNA was denatured with heat and formamide and reverse transcribed with either EHDV-1 or EHDV-2 outer primer pairs. The RT product was amplified by PCR, using 40 cycles of  $95^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 2 min, and 3 min in the final cycle. A sample was determined positive if a characteristic internal amplification product of predicted size (862 bp for EHDV-1 and 1,015 bp for EHDV-2) hybridized to specific DNA probes (below). Purified cDNAs from EHDV-1 and EHDV-2 were used respectively as positive and negative PCR controls. Additional controls consisted of sterile water and erythrocyte lysates from deer negative for EHDV by serology.

Amplification products were resolved by electrophoresis in 2% agarose gels, blotted to Gene-Screen Plus nylon membranes (DuPont NEN, Wilmington, Del.) for 18 h, and then baked for 2 h at  $80^{\circ}\text{C}$  under negative pressure. The blots were hybridized with digoxigenin (DIG)-11-dUTP-labeled DNA probes (300 to 500 bp) generated by random priming (Boehringer Mannheim, Indianapolis,

Ind.) of cDNA derived from cloned EHDV-1 or EHDV-2 gene segment 6. The probes were heated to  $95^{\circ}\text{C}$  for 10 min and then rapidly cooled to  $4^{\circ}\text{C}$  and 0.5 ml (0.2 ng of DNA/ $\mu\text{l}$  of 1:1 hybridization mix and formamide) applied to the prehybridized membrane. Hybridization was carried out at  $56^{\circ}\text{C}$  for 12 h in a hybridization oven (Hybaid, Woodbridge, N.J.). The membranes were then washed twice in  $1\times$  SSC (0.15 M NaCl plus 0.015 M sodium citrate) for 10 min at  $37^{\circ}\text{C}$ , once for 10 min in  $1\times$  SSC at  $60^{\circ}\text{C}$ , and once in  $2\times$  SSC at  $42^{\circ}\text{C}$  for 10 min and then air dried. After blocking for 30 min in 10% sheep serum and 5% deer serum, the membranes were washed and reacted with biotinylated sheep anti-DIG antibody (Boehringer Mannheim) for 30 min, then with streptavidin for 30 min, and finally with 3',3'-diaminobenzidine (DAB; Vector Laboratories, Burlingame, Calif.) as the chromogen. Hybrids were identified by a brown precipitate. EHDV-1- and EHDV-2-infected and uninfected cattle pulmonary arteriole endothelial (CPAE) cells, and DIG-labeled DNA probes (300 to 500 bp) complementary to bluetongue virus (BTV), a closely related orbivirus, gene segment 6 (nonspecific but closely related cDNA) and DIG-labeled plasmid DNA (nonsense probe), were used as controls for all hybridization reactions.

**Antiviral antibody.** Peripheral blood and/or heart blood was collected in K<sub>2</sub>EDTA. Plasma was separated from cells by centrifugation and stored frozen at  $-80^{\circ}\text{C}$ . Thawed plasma was assessed for antiviral antibody to purified EHDV and BTV by agar gel immunodiffusion (AGID) tests and radioimmunoprecipitation assay (RIPA) (Fig. 1). AGID tests were performed with commercial kits that detect precipitating antibodies to both EHDV and BTV (Veterinary Diagnostic Technology, Wheat Ridge, Colo.). RIPAs were done according to previous published procedures for closely related orbiviruses (33). Briefly, BHK-21 cells were infected with EHDV-1 or EHDV-2 at 1 PFU/cell and at 18 to 24 h following infection were labeled with 50  $\mu\text{Ci}$  of [<sup>35</sup>S]methionine (New England Nuclear, Boston, Mass.) per ml in minimal essential medium (without methionine) containing 2% fetal bovine serum. Cells were lysed with RIPA buffer (29) containing 1% Triton X-100 and 0.1% aprotinin (Sigma) and centrifuged at  $15,000 \times g$  for 5 min. The cell lysates were immunoprecipitated by using antiserum produced in rabbits against each of the two U.S. EHDV serotypes. *Staphylococcus aureus* protein A (Pansorbin; Calbiochem, La Jolla, Calif.) was used as the solid phase in the reaction. The immunoprecipitated viral proteins were eluted from the Pansorbin by boiling in polyacrylamide gel electrophoresis (PAGE) sample buffer. The samples were then analyzed by sodium dodecyl sulfate (SDS)-PAGE and autoradiography. In addition, gel filtration chromatography was used to separate plasma antibody species. Briefly, plasma was loaded to columns containing Sephadex G-25F (medium size) as recommended by the manufacturer (Pharmacia Biotech Inc., Piscataway, N.J.). The excluded fraction, containing putative immunoglobulin M (IgM), was further purified by ammonium sulfate precipitation and was compared to the eluted fraction (putative IgG) by RIPA. Extracts from uninfected BHK-21 cells were immunoprecipitated with the same antiserum and used as controls to distinguish cellular proteins from viral proteins.

**Virus isolation.** Monolayer cultures (25 cm<sup>2</sup> tissue culture flasks) of CPAE cells (ATCC CCL-209) were inoculated with peripheral blood erythrocyte lysate

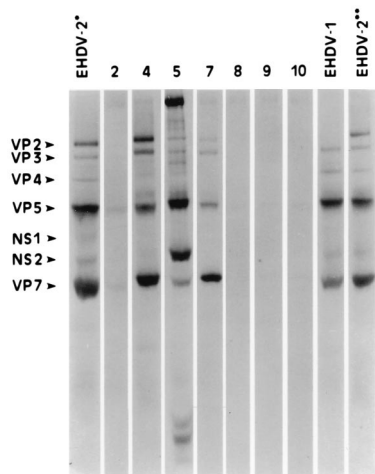


FIG. 1. Antibodies to structural and nonstructural proteins of EHDV. Viral proteins of EHDV-1 (Indiana) and EHDV-2 (Alberta), the only two serotypes found in the United States, were labeled with [ $^{35}$ S]methionine, reacted with sera (1:100 dilution) from deer, antelope, and domestic ruminants suspected of EHDV infection or having contact with infected animals, and analyzed by SDS-PAGE and autoradiography. Lanes: EHDV-1, EHDV-2\* (isolate 1), and EHDV-2\*\* (isolate 2), viral polypeptides reacted with serotype-specific antisera raised in experimentally infected rabbits; 2, 4, and 7, test sera from white-tailed deer; 5, test serum from a pronghorn antelope; 8 to 10, sera from clinically healthy, lesion-free, EHDV PCR-negative deer from the affected area, none of which showed precipitating antibodies to EHDV-2.

( $10^9$  erythrocytes), blood monocytes, and/or  $0.5 \text{ cm}^3$  of homogenized tissue. Briefly, leukocytes were removed from blood by density gradient centrifugation and monocytes were separated by adherence to plastic surfaces as described previously (6). The erythrocyte fraction was then diluted to original volume in Hanks' balanced salt solution, and the cell suspension was added to 9 volumes of sterile water and incubated for 2 h at  $4^\circ\text{C}$  to induce erythrolysis. Erythrocyte membranes were collected by centrifugation ( $500 \times g$ ). Solid tissues were prepared for coculture as described previously (9). The cultures were maintained in minimal essential medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah),  $1 \times$  nonessential amino acids,  $5 \times 10^{-6} \text{ M}$  2-mercaptoethanol, 2 mM L-glutamine, and 50  $\mu\text{g}$  of gentamicin (Sigma) per ml. CPAE monolayers were observed daily for cytopathic effects, passaged at 6-day intervals, and maintained for a minimum of 18 days or until cytopathic effects were observed. The cells were then harvested (1% trypsin and sodium EDTA) and cytocentrifuged to glass microscope slides (Superfrost Plus; Fisher Scientific, Pittsburgh, Pa.) and/or transferred to fibronectin (20  $\mu\text{g}/\text{ml}$ )-coated four-quadrant glass chamber slides (Nunc Inc., Naperville, Ill.) for 24 h. The slides were treated with 100% acetone for 10 min at  $4^\circ\text{C}$ , air dried, and saved frozen ( $-80^\circ\text{C}$ ).

For immunocytochemical detection of EHDV, the cytocentrifuge or chamber slide preparations were rehydrated in phosphate-buffered saline (PBS) and then reacted with a monoclonal antibody (MAb; 20  $\mu\text{g}/\text{ml}$ ) to the EHDV inner capsid protein VP7 (hybridoma 11C6.288, IgG1 isotype, affinity purified). After 1 h at room temperature, the slides were washed, rinsed, and air dried. A goat anti-mouse IgG was added and incubated for 1 h at room temperature. After washing in PBS, an avidin-biotin-peroxidase complex (ABC Vectastain kit; Vector Laboratories) was added and incubated for 1 h at room temperature. The slides were then washed in PBS, and DAB substrate solution (20 mg/ml) was added. The remainder of the procedure was as described previously (6, 9).

For indirect fluorescent-antibody staining, acetone-fixed cytospin preparations were rehydrated in PBS for 10 min at room temperature, air dried, and stained for 1 h at room temperature in a humid chamber with an anti-VP7 MAb. Slides were then washed three times in PBS, rinsed in distilled water, and air dried. Fluorescein-conjugated goat anti-mouse IgG was added and incubated for 1 h at room temperature. The slides were washed in PBS, and coverslips were mounted in PBS-glycerol (1:9). The slides were examined with an epifluorescence microscope equipped with a 150-W xenon burner and an IF-490 exciter filter.

**Gross and microscopic pathology.** Animals were necropsied in the field, and gross lesions were identified (Fig. 2A and B). Formalin-fixed, paraffin-embedded tissues were sectioned (5  $\mu\text{m}$ ) by routine methods, stained with hematoxylin and eosin, and examined by light microscopy (Fig. 2C to F).

**Immunohistochemistry.** A MAb to EHDV VP7 (11C6.288; 20  $\mu\text{g}/\text{ml}$ ) was applied to a wide variety of cryostat-sectioned snap-frozen tissues. The procedure used an ABC peroxidase technique with DAB as the chromogen (Vector Laboratories) and was performed by methods described previously (7, 8). Irrel-

evant MABs (IgG1) to bovine herpesvirus 1 glycoprotein, EHDV-2-infected and uninfected CPAE, and a variety of tissues from deer that were negative for EHDV by PCR were used as controls.

**In situ hybridization.** Tissue sections were deparaffinized and then rehydrated in Tris-buffered saline (TBS; 0.1 M Tris [pH 7.5], 0.1 M NaCl), digested with proteinase K (20  $\mu\text{g}/\text{ml}$ ,  $37^\circ\text{C}$ ; Sigma) for 60 min at room temperature, and washed in diethyl pyrocarbonate-treated water. The sections were then treated with RT mix (as described for PCR), coverslipped, and incubated for 1 h at  $37^\circ\text{C}$ . Full-length copies of the coding region of gene segment 6 from EHDV-2 (1,803 bp; GenBank accession no. L27647) and BTv-10 (1,769 bp; GenBank accession no. Y00422) were cloned into transcription vectors under the control of T7 and SP6 RNA polymerase promoters (pGEM [EHDV-2; Promega]; pCR II [BTv-10; Invitrogen, San Diego, Calif.]). The constructs were linearized with the restriction enzymes and used as templates for transcription reactions utilizing T7 and SP6 polymerase and DIG-conjugated UTP (DIG RNA labeling mix; Boehringer Mannheim). The resulting transcripts were precipitated with ethanol, hydrolyzed into smaller fragments, reprecipitated, and quantified spectrophotometrically. The specificity and working concentrations of the final products were determined by dot blot analysis (8). The probes (sense and antisense) were then applied to the tissues at a final concentration of 5 ng in a solution containing hybridization mix and formamide at 1:1. Hybridization was performed overnight at  $42^\circ\text{C}$  in a humidified chamber. Following incubation, the slides were rinsed and blocked (nucleic acid blocking reagent; Boehringer Mannheim). Horseradish peroxidase-conjugated Fab fragments from anti-DIG antibody raised in sheep (Boehringer Mannheim) were diluted 1:50 (3,000 mU/ml) in TBS and applied to tissues for 30 min at room temperature. The slides were then washed in TBS for 2 min, and DAB substrate added for 5 to 10 min as instructed by the manufacturer of the kit (Vector Laboratories). The presence of viral nucleic acid was indicated by a brown cell-associated precipitate.

Cytospin preparations of EHDV-2-infected and uninfected cultured CPAE cells, as well as tissues from deer that were negative for EHDV by PCR, were run consecutively as controls. Experiments were also performed to evaluate the sensitivity of RNA probes used for in situ hybridization. Cultured CPAE cells were infected with EHDV-2 at multiplicities of infection (MOIs) of 0.1, 1, and 10 for 1, 4, 12, and 24 h as previously described (23). The cells were then fixed in a nonaldehyde, non-cross-linking, water-soluble fixative (Permafix; Ortho Diagnostics, Raritan, N.J.), suspended in plasma clots (15), and embedded in paraffin, and sections containing known copy numbers of virus were reacted with specific riboprobes. Using this technique, cells containing  $\geq 20$  virus copies could be detected. Hybridization controls consisted of reacting tissues with nonsense RNA probes to the human immunodeficiency virus (HIV) type 1 *gag* gene (DIG-HIV *gag* RNA).

**RT in situ PCR.** Following deparaffinization, tissue sections were rehydrated in TBS, protease digested, washed in diethyl pyrocarbonate-treated water, and incubated overnight at  $37^\circ\text{C}$  in an RNase-free DNase I solution (Boehringer Mannheim). The sections were reacted with RT mix according to the manufacturer's recommendations (RT-PCR kit; Perkin-Elmer, Norwalk, Conn.), which included heating to  $70^\circ\text{C}$  for 2 min followed by a 50-min incubation at  $42^\circ\text{C}$ . Next, a solution containing PCR buffer (50 mM KCl, 10 mM Tris HCl [pH 8.3]), 4 mM  $\text{MgCl}_2$ , 0.01% gelatin, 200  $\mu\text{M}$  deoxynucleoside triphosphates, 50 pM each primer, and *Taq* polymerase (0.15 U/ $\mu\text{l}$ ) was made. The mixture was then added to sections in volumes that ranged from 30 to 50  $\mu\text{l}$ , depending on the size of the section, and coverslipped. Because the EHDV genome is double stranded, both 5' (E2N1-N2-5, AGCATTATCACCACAGTGGACGTG) and 3' (E2N1-N2-3, AGCCATAGCCTGAGCGATGTTTCAT) internal primers were used in the RT reactions and PCRs. To prevent evaporation, coverslips were anchored with nail polish and edges were covered with mineral oil. The slides were then placed directly on the aluminum block of the thermocycler (OmniGene; Hybaid), and humidity covers were applied. After 25 cycles of denaturation at  $94^\circ\text{C}$  for 1 min and annealing at  $55^\circ\text{C}$  for 2 min, followed by polymerization for 2 min at  $72^\circ\text{C}$ , the slides were removed, treated for 5 min with xylenes to remove mineral oil and for 5 min in 100% ethanol, and then air dried. The anticipated PCR product was 1,573 bp and represented 87% of the EHDV-2 gene segment 6. Amplified DNA was detected by hybridization to DIG-labeled RNA probes specific for EHDV-2 gene segment 6 and were shown previously to hybridize internal to the PCR primer binding sites. The remainder of the procedure was as described for in situ hybridization, including controls.

## RESULTS

**Antibody response to infection.** All deer and antelope demonstrating clinical and/or pathological signs of infection and disease had plasma antibody to EHDV-2 by RIPA (Fig. 1). Gel filtration chromatography showed that the predominant antibody species in plasma was IgM. In fact, only one animal (no. 2) showed a weak IgG response and demonstrated precipitating antibody by AGID (Table 2). Collectively, these findings suggest that plasma antibody concentrations to EHDV were low (i.e., lack of precipitating antibody by AGID) and repre-



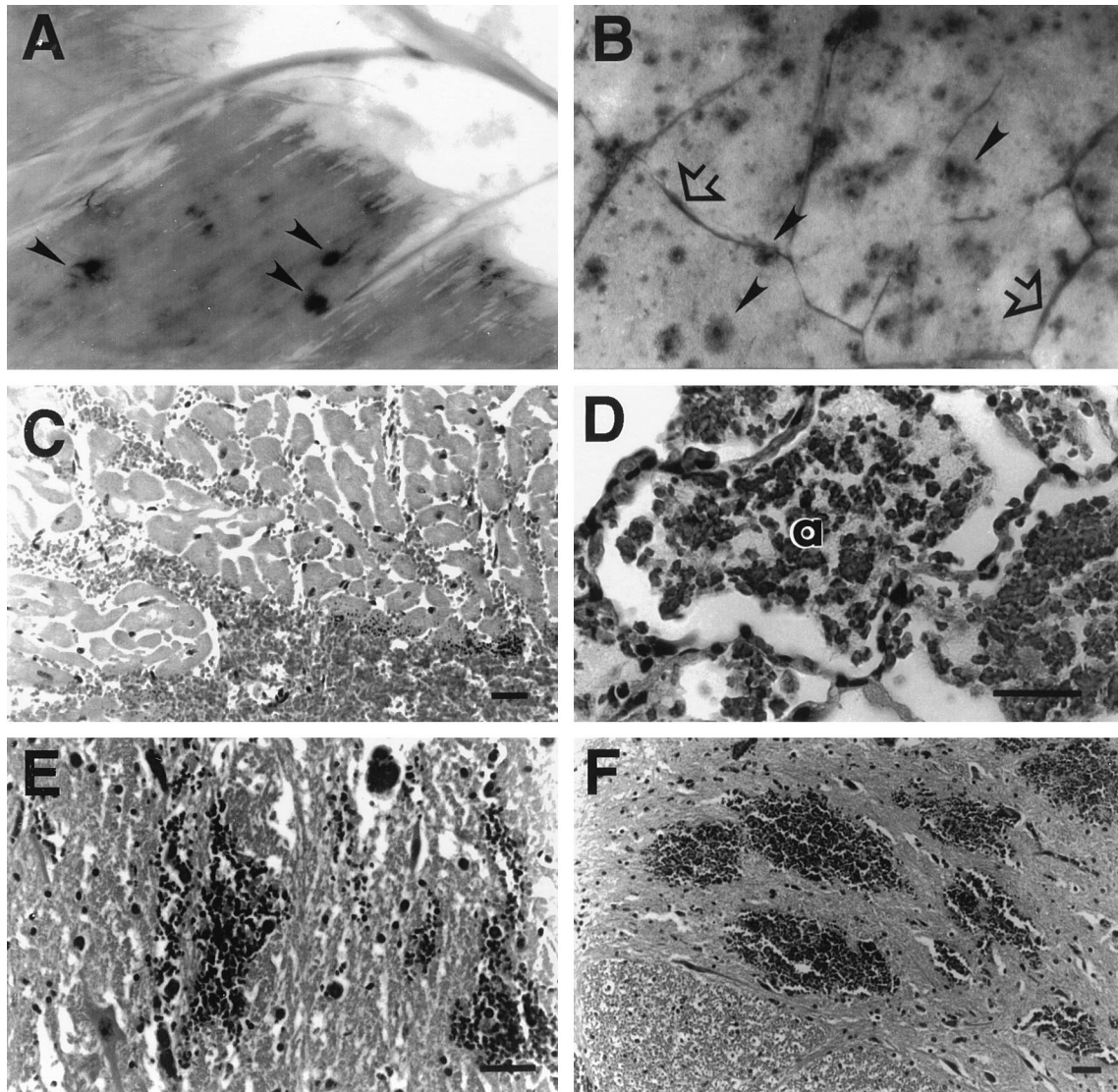


FIG. 2. Representative gross (A and B) and microscopic (C to F) lesions associated with laboratory-confirmed EHDV-2 infection of white-tailed deer and pronghorn antelope. (A) Heart, epicardial surface showing multifocal petechial hemorrhages (single arrows) (no. 7; magnification,  $\times 2$ ). (B) Lung, pleural surface of caudal lobe showing multifocal petechial hemorrhages (single arrows), widespread congestion, and interlobular edema and emphysema (open arrows) (no. 5; magnification,  $\times 2$ ). (C) Acute myocardial inter-fibrillar hemorrhage (no. 5). (D) Pulmonary intra-alveolar (a) hemorrhage (no. 7). (E) Acute hemorrhage and edema in the cerebral cortex (no. 7). (F) Acute multifocal hemorrhage in the cervical spinal cord (no. 4). (C to F) Hematoxylin and eosin; bar = 100  $\mu\text{m}$ .

sented a primary antibody response to infection. This conclusion is corroborated by clinical and pathological findings of rapid progression from infection to death and widespread hemorrhage and ischemic necrosis with little or no evidence of inflammation or altered hematopoiesis.

The antibody profiles of seropositive white-tailed deer (no. 2, 4, and 7) and of the pronghorn antelope (no. 5) were markedly different (Fig. 1). The antelope reacted strongly to EHDV-2 inner capsid protein VP7, nonstructural protein 2 (NS2), and several minor proteins. In contrast, deer responded strongly only to VP7. Most animals infected with EHDV-2 also demonstrated antibody to outer capsid proteins VP2 and VP5. This was in contrast to plasma from animals infected with EHDV-1 (USDA-ARS-ABADRL reference reagent), to which there was no reactivity to VP2. The significance of differential reactivity to structural and nonstructural proteins

with respect to orbiviral pathogenicity and host immune response is not known.

There was no evidence of antibodies to EHDV or related orbiviruses in plasma collected from clinically normal deer or from domestic livestock that were surveyed within the geographic boundaries of the epizootic and from which samples were collected several months following the first confirmed case of EHDV-2. Furthermore, RNA preparations from erythrocyte lysates showed that these animals were negative for EHDV and BTV by PCR.

**Clinicopathologic features.** Because the time frame from infection to death was short, very few animals were observed to have clinical signs attributable infection with EHDV. When present, clinical signs included disorientation, lethargy, and bleeding from oronasal, rectal, and/or urogenital cavities. Animals with signs of hemorrhagic fever usually died within 24 to

TABLE 2. Laboratory findings<sup>a</sup>

Animal no.	VI <sup>b</sup>	IFA/IP <sup>c</sup>	PCR specific for <sup>d</sup> :			EHDV-specific AGID	EHDV-2-specific RIPA	EHDV-2-specific RT in situ PCR
			EHDV-1	EHDV-2	BTV			
1	+	+	—	+	—	—	+	+
2	—	+	—	+	—	+	+	+
3	—	+	—	+	—	—	+	+
4	+	+	—	+	—	—	+	+
5	+	+	—	+	—	—	+	+
6	+	+	—	+	—	—	+	+
7	+	+	—	+	—	—	+	+
8	—	—	—	—	—	—	—	—
9	—	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—

<sup>a</sup> Animals 8 to 10 represent clinically healthy, lesion-free deer and antelope from the affected area. Antiserum to BTV was cross-reactive with BTV serotypes 2, 10, 11, 13, 17, and 20.

<sup>b</sup> VI, virus isolation. Erythrocyte lysates and/or tissue homogenates were inoculated into embryonated chicken eggs and/or CPAE cells.

<sup>c</sup> IFA/IP, indirect immunofluorescence (IFA) and/or immunoperoxidase (IP) stain. Fluorescein- or biotin-conjugated MAbs specific for EHDV (hybridoma 11C6.288) or BTV (hybridoma 1004.90) inner capsid protein (VP7) were reacted with CPAE cells which had been cocultivated with clinical samples. MAbs were provided as a gift by Michael Jochim, Veterinary Diagnostic Technology, Inc., Wheat Ridge, Colo.

<sup>d</sup> Erythrocyte-associated orbiviral nucleic acid was detected by RT nested PCR using nucleotide primers specific for EHDV-1 and EHDV-2 (1, 2, 58) or BTV (all North American serotypes) (57).

48 h following first observation. Upon gross examination frank blood or large volumes of sanguinous fluid were present in the pericardial sac (100 to 200 ml) and in the thoracic cavity from most clinically affected animals and from all animals found dead and diagnosed with EHDV-2 infection (Table 1). Petechial and/or ecchymotic hemorrhages were typically present at the base of the pulmonary artery and on the epicardial surface of the heart (Fig. 2A). The lungs of affected animals were heavy (edema) and darkened (cyanosis). Multifocal petechial and/or ecchymotic hemorrhage and interlobular edema were visible on the pleural surfaces (Fig. 2B), and a sanguinous froth was present in the trachea. In addition, most animals had evidence of edema, hyperemia, and petechial and/or ecchymotic hemorrhages in the brain, spinal cord, trachea, rumen and/or abomasal mucosa, liver, gallbladder, urinary bladder, kidney, and lymph nodes. The spleen of most animals was pale and of reduced size, suggesting contraction.

Histopathological evaluation revealed acute multifocal basilar epithelial necrosis with little or no inflammation and mild acanthosis at mucocutaneous junctures including the lips, nares, coronary band, and rectum of all infected animals. In addition, severe and widespread edema, congestion, hemorrhage, and segmental necrosis, associated with microvascular thrombosis, were present in the subcutis, intramuscular fascial planes, smooth, cardiac (Fig. 2C), and skeletal muscles of most animals with confirmed EHDV-2 infection. Fragmented and hypertrophic muscle fibers were observed in the esophagus, tongue, neck, and myocardium. Coagulative necrosis of the papillary muscle of the left ventricle was a common finding, as well as petechial hemorrhage in the tunica media at the base of the pulmonary artery (no. 2, 4, 5, and 7), which was visible from the adventitial and luminal surfaces. Similarly, widespread edema, congestion, and/or hemorrhage were present in the larynx, trachea, thyroid, lung (Fig. 2D), brain (Fig. 2E), spinal cord (Fig. 2F), liver, kidney, urinary bladder, rumen mucosa (papillae and pillars), mucosal surface of the reticulum (plicae), abomasal mucosa, small and large bowel (mucosal and serosal surfaces), and most lymph nodes. Small thin-walled vessels in areas of hemorrhage and necrosis were often occluded by fibrin and platelet aggregates. Glomerular hyaline thrombi, interglomerular and intertubular hemorrhage, and acute renal tubular necrosis were also observed. Splenic contraction was a common finding and was manifested histologi-

cally by a loss of erythrocytes in erythrocyte-dependent areas (red pulp). Analysis of bone marrow revealed normal numbers of megakaryocytes, as well as erythroid and myeloid precursors.

**Isolation and typing of viruses.** Infectious EHDV was isolated and/or viral RNA was detected in peripheral blood erythrocyte lysates inoculated into CPAE culture from 7 of 10 animals examined (Table 2). Cytospin and/or chamber slide preparations were made when cytopathic effects were first observed and in all cultures by 18 days following inoculation. Cell preparations were then assessed for EHDV and BTV protein antigens (VP7) by indirect immunofluorescence and/or immunoperoxidase staining. Cell culture from animals demonstrating clinical signs and/or pathology consistent with EHDV infection were all positive by immunofluorescent and/or immunoperoxidase staining procedures (Table 2). In addition, EHDV-2 was isolated and/or viral RNA was detected by use of serotype-specific PCR from homogenates of brain, spinal cord, lung, heart, lymph node, uterus, liver, kidney, rumen, skeletal muscle (tongue), and skin (lingual-facial) (data not shown). Infectious virus and viral nucleic acid was also detected in pericardial fluid from all animals with cardiac lesions, and viral RNA was shown to be present in cells lining the pericardium by RT in situ PCR. All virus isolates were shown to be EHDV-2 by serotype-specific PCR (Table 2) and by RIPA (Fig. 1).

**Tissue distribution and cellular localization of viral proteins and nucleic acids.** Tissues from which virus was isolated by cocultivation were snap-frozen, and cryostat sections were shown to be negative for expression of EHDV capsid antigen by immunohistochemistry. Similarly, only occasional cells in animals with severe lesions (no. 4 and 5) showed viral RNA by in situ hybridization, and only in selected tissues such as lung and lymph node. In contrast, viral RNA was detected by RT in situ PCR in large numbers of mononuclear leukocytes within the lymph node (Fig. 3A), tonsil (Fig. 3B), lung (Fig. 3C), skin, and bone marrow and in capillary and arteriole endothelial cells within the lung (Fig. 3D), cerebrum (Fig. 3E), spinal cord, heart (Fig. 3F), and pericardial sac. Cell-associated virus in lymphoid tissues localized mainly within the medullary sinuses and to cells morphologically compatible with macrophages. Similarly, virus-positive cells in the lung were found within the pulmonary interstitium in sites where macrophages typically reside. EHDV was detected in tissues with multifocal hemor-



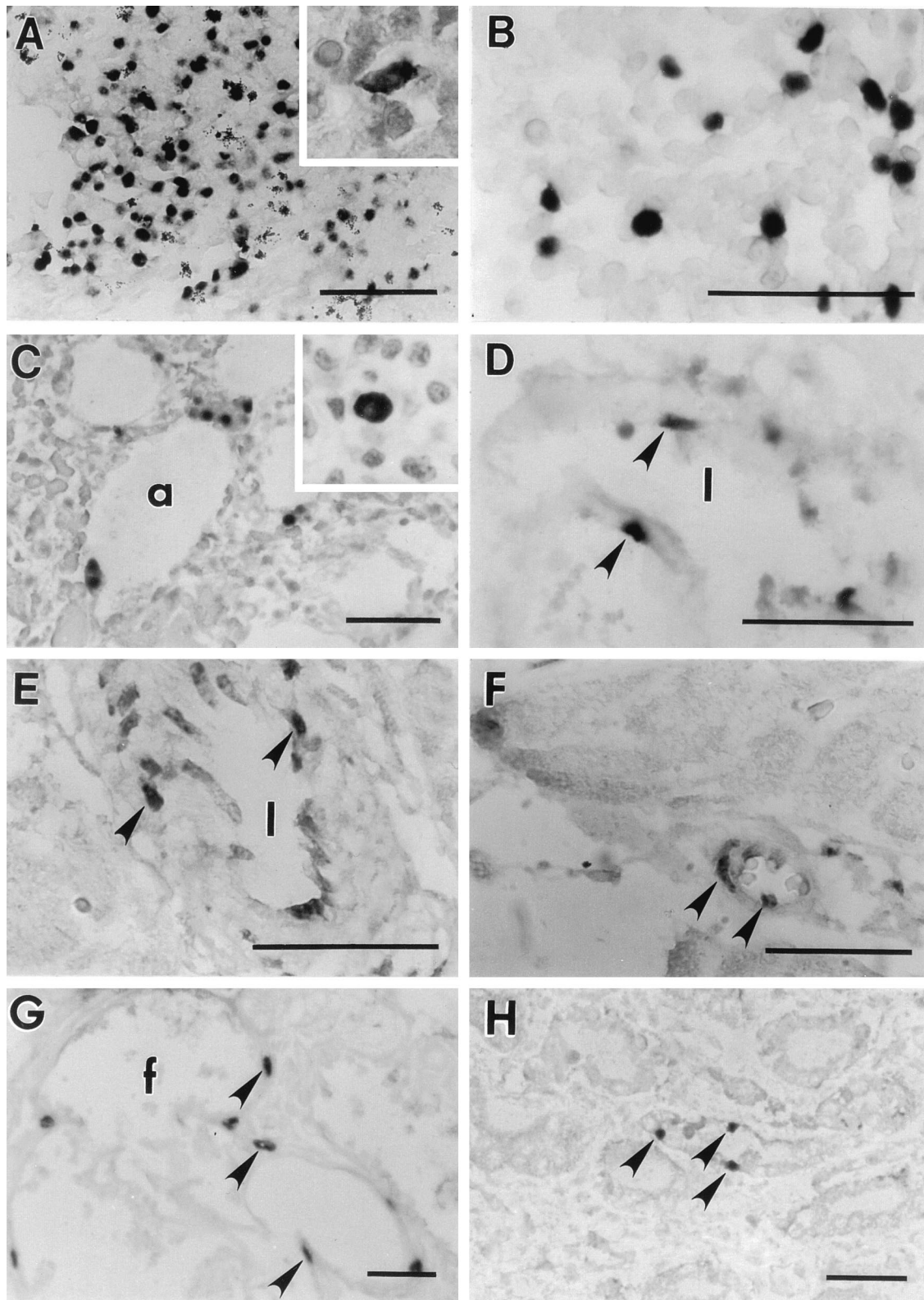


FIG. 3. Intracytoplasmic localization of EHDV-2 (arrows) by RT-PCR-driven in situ hybridization. Virus localized to mononuclear leukocytes in a wide variety of tissues, including mediastinal lymph node (A; no. 3), palatine tonsil (B; no. 5), and lung in sites adjacent to alveoli (a) (C; no. 4), and to vascular endothelium in tissues with hemorrhage attributable to microvascular degeneration. Pictured is a thin-walled pulmonary venule (D; no. 2), small artery in the cerebrum (E; no. 4), and a capillary in the myocardium (F; no. 7) to which EHDV RNA was localized to cells lining the vascular lumen (l). Viral transcripts were also found within cells lining hair follicles (f) in areas of erosion and ulceration (G; no. 5) and within renal tubular epithelium (H; no. 6) in regions of acute renal tubular necrosis. Staining was predominantly extranuclear (insets, A and C; magnification,  $\times 200$ ), as would be expected with an RNA virus having a cytoplasmic replication cycle. Alkaline phosphatase; bar = 100  $\mu\text{m}$ .

rhage; however, viral RNA was also found, but less frequently, in histologically normal tissue in association with vascular endothelium and/or resident mononuclear leukocytes. Viral transcripts were also found within cells lining hair follicles in areas of erosion and/or ulceration and vesicle formation (Fig. 3G) and within renal tubular cells (Fig. 3H) in areas of acute renal tubular necrosis. Staining was predominantly extranuclear (insets, Fig. 3A and C), as would be expected with an RNA virus having a cytoplasmic replication cycle. Omitting PCR resulted in a much reduced or absent hybridization signal.

Experiments using cell culture infected with EHDV-2 of specific MOI showed that cells containing as few as 20 virus particles could be detected by in situ hybridization, and as few as one to two virus copies were detected by RT in situ PCR. Collectively, these results show that RT in situ PCR was specific for EHDV-2 and that virus burden was generally very low (i.e., <20 virus copies per cell), yet infection was widespread and high numbers of individual cells were infected. The non-complementary BTV RNA and nonsense HIV RNA probes did not hybridize EHDV-infected cells or tissue, nor was non-specific hybridization to uninfected cell culture and uninfected tissue observed. In addition, peripheral blood thrombocytes that were obtained from an EHDV-negative deer and inoculated in culture with EHDV-2 at an MOI of 10 showed no evidence of internalized viral RNA by RT in situ PCR.

**Epizootiology.** Peripheral blood was collected from domestic cattle and sheep within the geographic boundaries of the epizootic no sooner than 2 months following the first confirmed case of EHDV-2 infection in deer. There was no evidence of antiviral antibodies to EHDV and BTV or of erythrocyte-associated orbiviral nucleic acids by PCR. However, relatively few animals were available for survey ( $n = 36$ ). Thus, the importance of domestic ruminants as a reservoir host for EHDV is yet uncertain, but limited data suggest that they did not have a substantial role in maintaining the virus during this outbreak in wildlife.

Among the few flying insects captured within the epizootic boundaries were *Culicoides* spp., which are known to be the primary arthropod vectors of BTV and will also transmit EHDV (48, 56). Although only two separate attempts at insect collection were made, numerous culicoid larval forms were found in soil with high organic content surrounding slow-running rivers and stagnant ponds: locations where dead animals were frequently found. Neither infectious EHDV or EHDV RNA was detected in larvae. We have shown previously that transovarial infection is not a mode of orbivirus transmission (37). Thus, the mode of virus transmission in the 1995 EHDV outbreak was not established. However, the nature of the outbreak, rapid spread over long distances, suggests that an arthropod vector was involved.

## DISCUSSION

EHDV and related orbiviruses comprise a group of arthropod-borne RNA viruses that are capable of rapid genotypic and phenotypic change and, at present, are mostly confined to animals. Considerable variation can occur in the severity and types of disease manifestations associated with orbivirus infection. Host and viral factors, as well as environmental circumstances under which the virus and host interact, can influence the outcome of infection. In North America, white-tailed deer, black-tailed deer (mule deer), pronghorn antelope, and elk are susceptible to infection. However, white-tailed deer are most severely affected. The rate of survival is much higher among mule deer and pronghorn antelope, and elk are only mildly affected (24, 45). Although epizootic hemorrhagic disease can

manifest as a subclinical or mild ulcerative disease in all the aforementioned species (24, 60), EHDV-2-infected white-tailed deer and antelope most frequently developed an acute and fatal hemorrhagic syndrome during the 1995 outbreak. Of particular interest was the magnitude of hemorrhage in the central nervous system. Hemorrhages in the brain and cervical spinal cord were widespread and may have contributed to the commonly observed signs of depression and disorientation. The extent of these neurologic lesions may also be due in part to a highly susceptible and immunologically naive animal population and/or the emergence of a virus variant with increased neurovirulence. Others have shown that the ability of orbiviruses to infect endothelial cells from distant anatomic sites is related to the virulence of a given virus isolate (31). Viral factors such as RNA polymerase infidelity and genome segment reassortment undoubtedly contribute to orbivirus heterogeneity (12, 19, 25), and salivary gland and midgut proteins of the arthropod host may also enhance orbivirus virulence (40, 48).

We have previously shown significant geographic variation in the genes encoding VP2 and VP3 from variants of EHDV-2 (13, 14). Others have shown similar variation with the gene encoding VP7 (41). In contrast, the genes encoding NS1 (22, 58), NS2 (35, 59), and NS3 (28) are highly conserved between isolates of EHDV-1 and -2 and are quite distinct from their BTV counterparts (22, 51, 52). NS1 is the product of gene segment 6 and is the major protein synthesized in orbivirus-infected cells (27). Furthermore, the mRNA coding for this protein is transcribed at a higher molar ratio than that of other orbiviral genes (26). Thus, gene segment 6 has been used as a target for RT-PCR. A comparison of these reports suggested that gene segment 6 would also be an ideal target for RT in situ PCR. Conversely, specific regions of gene segment 2 have been used to differentiate EHDV-1 from EHDV-2 and from other closely related orbiviruses (1, 2).

In contrast to peripheral blood erythrocytes, where virus and viral RNA can aggregate on the cell surface, orbiviral antigens and viral nucleic acid have been difficult to detect in tissues by using immunohistochemistry and/or classical in situ hybridization techniques (16, 17, 44), possibly as a result of low numbers of virus-infected cells and/or low levels of virus replication in situ (16). In the present study, an RT in situ PCR procedure was developed and used to localize viral nucleic acid to single cells. The technique was useful in resolving low-frequency expression of EHDV RNA. By amplifying virus in tissues via PCR prior to in situ hybridization, large numbers of mononuclear leukocytes and endothelial cells, in a wide variety of tissues, were shown to harbor cytoplasmic EHDV RNA. The number of infected cells and intensity of the hybridization reaction were influenced by the specific tissue type. For example, tissues such as brain, heart, and lung, with well-developed vascular systems and/or resident leukocyte populations, had increased numbers of cells containing viral RNA. Not surprisingly, these tissues also demonstrated the most severe lesions. Because infected cells were rarely detected by in situ hybridization alone, the virus copy number was very low. In vitro infection studies showed that under optimal conditions, highly permissive cells containing  $\geq 20$  copies of EHDV-2 could be detected reliably by in situ hybridization. Collectively, these results suggest that infection of deer and antelope with EHDV-2 was massive, yet disease resulted from low copy numbers of virus. Recent studies utilizing in situ PCR in blood and/or lymph node from HIV-infected people have shown similar findings of widespread latent or weakly productive infections in the presence of severe disease (21). Because our control samples gave predicted results, including cytoplasmic



localization of viral RNA, and were in concordance with all other laboratory findings, mispriming events, nonspecific hybridizations, and other potential causes of false reactions were unlikely.

PCR-driven *in situ* hybridization has also been used to detect single gene copies of lentiviruses (30, 46) and human papillomaviruses (39) at single-cell resolution. In the present study, the finding of EHDV RNA in resident tissue leukocytes confirms previous reports that blood monocytes (20, 54) and possibly lymphocytes (20) support orbivirus infection *in vitro*. PBMC have also been shown to harbor infectious virus and/or express cell surface viral antigens *in vivo*, although cells demonstrating productive infection are rare, usually fewer than 1 to 5 per 300,000 mononuclear leukocytes (17). Similar observations were made in the present study. In addition to mononuclear leukocytes, cells with morphological and topographic characteristics of endothelium were shown to harbor cytoplasmic viral RNA. Others have shown by transmission electron microscopy that endothelia support EHDV replication (31, 50). Because amplification was, in general, essential for detecting virus-infected cells and localizing intracytoplasmic viral RNA, it is likely that virus replication *in vivo* was restricted by host factors, as shown with other RNA viruses (18). It is also possible that cells are permissive to virus entry but refractory to productive virus replication. This is a common feature of single-stranded RNA viruses (9). Last, because orbiviruses are unique in their association with thrombocytes (4), it was important to distinguish virus infection of endothelium from platelet aggregation to sites of vascular injury. EHDV mRNA localized exclusively within the cytoplasm of endothelial cells and never to the cell surface. This finding is consistent with ultrastructural studies of African horsesickness virus, an orbivirus closely related to EHDV, where viral particles were found only within endothelial cells and not within aggregates of thrombocytes and monocytes present on the endothelial cell surface (31). Furthermore, viral RNA was found in endothelial cells of EHDV-infected deer without histologic evidence of microvascular disruption, and intracellular virus could not be localized by RT *in situ* PCR in preparations of blood platelets.

The finding that most animals failed to develop acute inflammation, lacked evidence of altered myeloid or erythroid hematopoiesis, and had no detectable secondary antibody response to EHDV-2 suggested that the majority of infected animals survived for a minimum of 3 to 5 days following infection. In general, infected animals survived long enough to initiate a primary antibody response, manifest by detectable concentrations of plasma IgM, but died before 7 to 10 days and prior to detectable concentrations of IgG antibody. These findings are indicative of acute infection and were corroborated by the observation that numerous deer were found dead; however, clinically affected deer were rarely seen during the outbreak. This finding suggests a possible correlation between host immune response and survival. In infections with other viruses such as HIV and most animal lentiviruses, a positive correlation exists between disease state and antibody concentration (10). Although EHDV is not known to suppress humoral immunity, the virus can cause suppression of cell-mediated immunity as early as 6 days following infection (42). Immunosuppressive diseases are usually not associated with acute self-limiting viral infections; however, it was interesting that large numbers of bone marrow cells contained orbiviral RNA, yet there was little or no evidence of inflammation or a bone marrow response to infection in animals that died acutely. Thus, the effect of orbivirus infection on hematopoietic stem cells needs further investigation. Similarly, because numerous, mostly thin-walled vessels in tissues with evidence

of acute vascular necrosis and hemorrhage showed fibrin and platelet aggregation or hyaline thrombi, the role of disseminated intravascular coagulation in acute orbiviral disease also requires further investigation.

In the present study, we demonstrate that RT *in situ* PCR is a viable investigative approach for localizing EHDV-2 to specific cells and provides new insights into mechanisms of acute viral infection and disease where low levels of viral nucleic acid reside. The finding of widespread EHDV-2 infection, manifest by low copy numbers of leukocyte- and endothelium-associated virus, suggests that low levels of virus replication can result in vascular necrosis leading to severe hemorrhage and death.

#### REFERENCES

- Aradaib, I. E., J. W. McBride, W. C. Wilson, and B. I. Osburn. 1995. Development of polymerase chain reaction for specific identification of epizootic hemorrhagic disease virus serotype 1. *Arch. Virol.* **140**:2273–2281.
- Aradaib, I. E., W. C. Wilson, I. W. Cheney, J. E. Pearson, and B. I. Osburn. 1995. Application of PCR for specific identification of epizootic hemorrhagic disease virus serotype 2. *J. Vet. Diagn. Invest.* **7**:388–392.
- Bagasara, O., and R. J. Pomerantz. 1994. *In situ* polymerase chain reaction and HIV-1. *Clin. Lab. Med.* **14**:351–365.
- Barratt-Boyes, S. M., and N. J. MacLachlan. 1994. Dynamics of viral spread in bluetongue virus infected calves. *Vet. Microbiol.* **40**:361–371.
- Brewer, A. W., and N. J. MacLachlan. 1994. The pathogenesis of bluetongue virus infection of bovine blood cells *in vitro*: ultrastructural characterization. *Arch. Virol.* **136**:287–298.
- Brodie, S. J., K. A. Marcom, L. D. Pearson, B. C. Anderson, A. de la Concha-Bermejillo, J. A. Ellis, and J. C. DeMartini. 1992. The effects of virus load in the pathogenesis of lentivirus-induced lymphoid interstitial pneumonia. *J. Infect. Dis.* **166**:531–541.
- Brodie, S. J., L. D. Pearson, G. D. Snower, and J. C. DeMartini. 1993. Host-virus interaction as defined by amplification of viral DNA and serology in lentivirus infected sheep. *Arch. Virol.* **130**:413–428.
- Brodie, S. J., V. G. Sasseville, K. A. Reimann, M. A. Simon, P. K. Seghal, and D. J. Ringler. 1994. Macrophage function in simian AIDS: killing defects *in vivo* are independent of macrophage infection, associated with alterations in Th phenotype, and reversible with IFN- $\gamma$ . *J. Immunol.* **153**:5790–5801.
- Brodie, S. J., L. D. Pearson, M. C. Zink, H. M. Bickle, B. C. Anderson, K. A. Marcom, and J. C. DeMartini. 1995. Ovine lentivirus expression and disease: virus replication, but not entry, is restricted to macrophages of specific tissues. *Am. J. Pathol.* **146**:1–13.
- Brodie, S. J., H. M. Bickle, and J. C. DeMartini. 1995. Ovine lentivirus-associated subclinical encephalomyelitis: virological markers in cerebrospinal fluid are predictive of central nervous system disease. *Clin. Immunol. Immunopathol.* **77**:14–18.
- Brown, C. C., R. F. Meyer, and M. J. Grubman. 1993. Use of a DIG-labeled RNA probe to detect all 24 serotypes of bluetongue virus in cell culture. *J. Vet. Diagn. Invest.* **5**:159–162.
- Calisher, C. H. 1994. Medically important arboviruses of the United States and Canada. *Clin. Microbiol. Rev.* **7**:89–116.
- Cheney, I. W., M. D. Larson, J. O. Mecham, and W. C. Wilson. 1995. Geographical genetic variation in the gene encoding VP3 from the Alberta isolate of epizootic hemorrhagic disease virus. *Virus Res.* **36**:279–286.
- Cheney, I. W., M. Yamakawa, P. Roy, J. O. Mecham, and W. C. Wilson. 1996. Molecular characterization of the segment 2 gene of epizootic hemorrhagic disease virus serotype 2: gene sequence and genetic diversity. *Virology* **224**:555–560.
- Cottler-Fox, M., and C. H. Fox. 1991. Examining cells for infectious agents: a novel approach. *J. Infect. Dis.* **161**:1239–1240.
- Dangler, C. A., A. de la Concha-Bermejillo, J. L. Stott, and B. I. Osburn. 1990. Limitations of *in situ* hybridization for the detection of bluetongue virus in blood mononuclear cells. *J. Vet. Diagn. Invest.* **2**:303–307.
- de la Concha-Bermejillo, A., C. E. Schore, C. A. Dangler, C. C. de Mattos, C. A. de Mattos, and B. I. Osburn. 1992. Comparison of slot blot nucleic acid hybridization, immunofluorescence, and virus isolation techniques to detect bluetongue virus in blood mononuclear cells from cattle with experimentally induced infection. *Am. J. Vet. Res.* **53**:2245–2250.
- de la Concha-Bermejillo, A., S. J. Brodie, S. Magnus-Corral, R. A. Bowen, and J. C. DeMartini. 1995. Pathologic and serologic responses of isogenic twin lambs to phenotypically distinct lentiviruses. *J. Acquired Immune Defic. Syndr. Hum. Retrovirol.* **8**:116–123.
- Domingo, E., E. Martinez-Salas, F. Sobrino, J. C. de la Torre, A. Portela, J. Ortin, C. Lopez-Galindez, P. Perez-Brena, N. Villaneuva, R. Najera, S. VandePol, D. Steinhauer, N. DePolo, and J. Holland. 1985. The quasispecies (extremely heterologous) nature of viral RNA genome populations: biological relevance—a review. *Gene* **40**:1–8.
- Ellis, J. A., M. L. Coen, N. J. MacLachlan, W. C. Wilson, E. S. Williams, and



- A. J. Leudke. 1993. Prevalence of bluetongue virus expression in leukocytes from experimentally infected ruminants. *Am. J. Vet. Res.* **54**:1452-1456.
21. Embretson, J., M. Zupancic, J. Beneke, M. Till, S. Wolinsky, J. L. Ribas, A. Burke, and A. T. Haase. 1993. Analysis of human immunodeficiency virus-infected tissues by amplification and *in situ* hybridization reveals latent and permissive infections at single-cell resolution. *Proc. Natl. Acad. Sci. USA* **90**:357-361.
22. Grubman, M. J., and S. Samal. 1989. Nucleotide and deduced amino acid sequence of the nonstructural protein, NS1, of the U.S. bluetongue virus serotype 17. *Nucleic Acids Res.* **17**:10498.
23. Haase, A. T., E. F. Retzel, and K. A. Staskus. 1990. Amplification and detection of lentiviral DNA inside cells. *Proc. Natl. Acad. Sci. USA* **87**:4971-4975.
24. Hoff, G. L., and D. O. Trainer. 1981. Hemorrhagic diseases of wild ruminants, p. 45-53. In J. W. Davis, L. H. Karstad, and D. O. Trainer (ed.), *Infectious diseases of wild mammals*. Iowa State University Press, Ames, Iowa.
25. Holland, J. J., K. Spindler, F. Horodyski, E. Grabau, S. Nichol, and S. VandePol. 1982. Rapid evolution of RNA genomes. *Science* **215**:1577-1585.
26. Huismans, H., and D. W. Vervoerd. 1973. Control of transcription during the expression of the bluetongue virus genome. *Virology* **52**:81-88.
27. Huismans, H., and H. J. Els. 1979. Characterization of the tubules associated with the replication of three different orbiviruses. *Virology* **92**:397-406.
28. Jensen, M. J., and W. C. Wilson. 1995. A model for the membrane topology of the NS3 protein as predicted from the sequence of segment 10 of epizootic haemorrhagic disease virus serotype 1. *Arch. Virol.* **140**:799-805.
29. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115**:1617-1621.
30. Komminoth, P., A. A. Long, R. Ray, and H. J. Wolfe. 1992. *In situ* polymerase chain reaction detection of viral DNA, single copy genes, and gene rearrangements in cell suspensions and cytopins. *Diagn. Mol. Pathol.* **1**:85-97.
31. Laegreid, W. W., T. G. Burrage, M. Stone-Marschat, and A. Skowronek. 1992. Electron microscopic evidence for endothelial infection by African horsesickness virus. *Vet. Pathol.* **29**:554-556.
32. MacLachlan, N. J., R. A. Nunamaker, J. B. Katz, M. M. Sawyer, G. Y. Akita, B. I. Osburn, and W. J. Tabachnick. 1994. Detection of bluetongue virus in the blood of inoculated calves: comparison of virus isolation, PCR assay, and *in vitro* feeding of *Culicoides variipennis*. *Arch. Virol.* **136**:1-8.
33. Mecham, J. O., V. C. Dean, and M. M. Jochim. 1986. Correlation of serotype specificity and protein structure of the five U.S. serotypes of bluetongue virus. *J. Gen. Virol.* **67**:2617-2624.
34. Mecham, J. O., and V. C. Dean. 1988. Protein coding assignment for the genome of epizootic haemorrhagic disease virus. *J. Gen. Virol.* **69**:1255-1262.
35. Nel, L. H., L. A. Picard, and H. Huismans. 1990. A characterization of the nonstructural protein from which the virus-specific tubules in epizootic haemorrhagic disease virus-infected cells are composed. *Virus Res.* **18**:219-230.
36. Nettles, V. E., S. A. Hylton, D. E. Stallknecht, and W. R. Davidson. 1992. Epidemiology of epizootic hemorrhagic disease virus in the United States, p. 238-248. In T. E. Walton and B. I. Osburn (ed.), *Proceedings of the Second International Symposium on Bluetongue, African Horse Sickness, and Related Orbiviruses*. CRC Press, Boca Raton, Fla.
- 36a. Norelius, S. E. Personal communication.
37. Nunamaker, R. A., P. J. Sieburth, V. C. Dean, J. G. Wigington, C. E. Nunamaker, and J. O. Mecham. 1990. Absence of transovarial transmission of bluetongue virus in *Culicoides variipennis*: immunogold labelling of bluetongue virus antigen in developing oocytes from *Culicoides variipennis* (Coquillett). *Comp. Biochem. Physiol. Ser. A* **96**:19-31.
38. Nuovo, G. J., F. Gallery, P. MacConnell, J. Becker, and W. Bloch. 1991. An improved technique for the *in situ* detection of DNA after polymerase chain reaction amplification. *Am. J. Pathol.* **139**:1239-1244.
39. Nuovo, G. J., P. MacConnell, A. Forde, and P. Delvenne. 1991. Detection of human papillomavirus DNA in formalin-fixed tissues by *in situ* hybridization after amplification by polymerase chain reaction. *Am. J. Pathol.* **139**:847-854.
40. Perez de Leon, A. A., and W. J. Tabachnick. 1996. Apyrase activity and adenosine diphosphate induced platelet aggregation inhibition by the salivary gland proteins of *Culicoides variipennis*, the North American vector of bluetongue viruses. *Vet. Parasitol.* **61**:327-338.
41. Pritchard, L. I., A. R. Gould, W. C. Wilson, L. Thompson, P. P. C. Mertens, A. M. Wade-Evans. 1995. Complete nucleotide sequence of RNA segment 3 of bluetongue virus serotype 2 (ona-A). Phylogenetic analyses reveal the probable origin and relationship with other orbiviruses. *Virus Res.* **35**:247-261.
42. Quist, C. F., E. W. Howerth, D. I. Bounous, and D. E. Stallknecht. 1997. Cell-mediated immune response and IL-2 production in white-tailed deer experimentally infected with hemorrhagic disease viruses. *Vet. Immunol. Immunopathol.* **56**:283-297.
43. Schnittman, S. M., M. C. Psallidopoulos, H. C. Lane, L. Thompson, M. Baseler, F. Massari, C. H. Fox, N. P. Salzman, and A. S. Fauci. 1993. The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4. *Science* **245**:305-308.
44. Schoepp, R. J., C. D. Blair, P. Roy, and B. J. Beaty. 1991. Detection of bluetongue virus RNA by *in situ* hybridization: comparison with virus isolation and antigen detection. *J. Vet. Diagn. Invest.* **3**:22-28.
45. Shope, R. E., L. G. MacNamara, and R. A. Mangold. 1960. Virus induced epizootic hemorrhagic disease of the Virginia white-tailed deer (*Odocoileus virginianus*). *J. Exp. Med.* **111**:155-170.
46. Spann, W., K. Pachmann, H. Zabnienska, A. Piermeier, and B. Emmerich. 1991. *In situ* amplification of single copy gene segments in individual cells by the polymerase chain reaction. *Infection* **19**:242-244.
47. Tabachnick, W. J., N. J. MacLachlan, L. H. Thompson, G. J. Hunt, and J. F. Patton. 1996. Susceptibility of *Culicoides variipennis sonorensis* to infection by polymerase chain reaction-detectable bluetongue virus in cattle blood. *Am. J. Trop. Med. Hyg.* **54**:481-485.
48. Tabachnick, W. J. 1996. *Culicoides variipennis* and bluetongue-virus epidemiology in the United States. *Annu. Rev. Entomol.* **41**:23-43.
49. Thomas, F. C., N. Willis, and G. Ruckerbauer. 1974. Identification of viruses involved in the 1971 outbreak of hemorrhagic disease in southeastern United States white-tailed deer. *J. Wildl. Dis.* **10**:187-189.
50. Tsai, K., and L. Karstad. 1973. The pathogenesis of epizootic hemorrhagic disease of deer: an electron microscope study. *Am. J. Pathol.* **70**:379-391.
51. Unger, R. E., R. Y. Chuang, L. F. Chuang, B. I. Osburn, and R. H. Doi. 1988. The cloning of full-length genome segments 2, 5, 6 and 8 of bluetongue (BTV) serotype 17 and studies of their genetic relatedness to United States BTV serotypes. *Virology* **167**:296-298.
52. Urakawa, T., and P. Roy. 1988. Bluetongue virus tubules made in insect cells by recombinant baculoviruses: expression of the NS1 gene of bluetongue virus serotype 10. *J. Virol.* **62**:3919-3927.
53. Venter, E. H., G. J. Viljoen, L. H. Nel, H. Huismans, and A. A. van Dijk. 1991. A comparison of different genomic probes in the detection of virus-specific RNA in *Orbivirus*-infected cells. *J. Virol. Methods* **32**:171-180.
54. Whetter, L. E., N. J. MacLachlan, D. H. Gebhard, H. W. Heidner, and P. F. Moore. 1989. Bluetongue virus infection of bovine monocytes. *J. Gen. Virol.* **70**:1663-1676.
55. Wilhelm, A. R., and D. O. Trainer. 1967. A comparison of several viruses of epizootic hemorrhagic disease of deer. *J. Infect. Dis.* **117**:48-54.
56. Wilson, W. C. 1991. Detection of epizootic hemorrhagic disease virus in *Culicoides variipennis* (Diptera: Ceratopogonidae). *J. Med. Entomol.* **28**:742-744.
57. Wilson, W. C., and C. C. L. Chase. 1993. Nested and multiplex polymerase chain reactions in the identification of bluetongue virus infection in the biting midge *Culicoides variipennis*. *J. Virol. Methods* **45**:39-47.
58. Wilson, W. C. 1994. Development of a nested-PCR test based on sequence analysis of epizootic hemorrhagic disease viruses non-structural protein 1 (NS1). *Virus Res.* **31**:357-365.
59. Wilson, W. C. 1994. Sequence analysis of the non-structural protein 2 from epizootic hemorrhagic disease viruses. *Virus Res.* **34**:63-68.
60. Work, T. M., D. A. Jessup, and M. M. Sawyer. 1992. Experimental bluetongue and epizootic hemorrhagic disease virus infection in California black-tailed deer. *J. Wildl. Dis.* **28**:623-628.